Premating Behavior of Dealates of the Formosan Subterranean Termite and Evidence for the Presence of a Contact Sex Pheromone¹

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Swarming by alates of the Formosan subterranean termite, Coptotermes formosanus Shiraki, occurs around dusk during April-June in Louisiana and adjoining states. Dispersal is the primary purpose for swarming. We studied the premating behavior, following swarming. Loss of wings, especially in females, was essential for successful formation of tandem pairs between the two sexes. No calling was observed and apparently a long-range volatile sex pheromone, reported in some other species of termites, may not be involved in mate attraction in C. formosanus. Males moving randomly use their antennae to detect a female, and, together with maxillary and labial palps, contact is maintained with her while in tandem. Previously mated males paired with virgin females showed tandem behavior. The incidence was very low when both males and females were previously mated and then paired. Males with complete ablation of antennae did not form tandem pairs. Ablation of 10 terminal antennal segments or the labial palps caused a significant increase in the time to initiate tandem behavior. Electrophysiological recordings from gustatory sensilla on both antennae and maxillary palps revealed increased neural activity in response to female abdominal tip extracts compared to activity elicited

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by a solvent control. Analysis of extracts of male and female abdominal tips using high-performance liquid chromatography showed a peak unique to the female extract. We discuss the presence of a nonvolatile chemical in female C. formosanus and its involvement in the specific premating behavior.

KEY WORDS: contact sex pheromone; *Coptotermes formosanus*; swarming; tandem behavior; termites.

INTRODUCTION

The Formosan subterranean termite, Coptotermes formosanus Shiraki, accidentally introduced into the continental United States, and first detected during the early 1960s, has become established in several southern states (Woodson et al., 2001). It is a serious pest of wooden structures and even live trees and, together with the native subterranean species, causes over 2 billion in damage and control costs each year (Potter, 1997). The adult reproductive stage produces large swarms of alates from April through June in Louisiana (King and Spink, 1974). Ratcliffe et al. (1952) reported that a colony of a related species, Coptotermes lacteus (Froggatt), totaling about 1 million individuals, might produce 60,000 alates (6%) a year. Nutting (1969) suggested that the production of alates in most termites is controlled by pheromones and nutrients. Colony age, which is a measure of its size or maturity, appears to be a fundamental determinant in alate production. Alates show positive phototaxis and generally fly to the nearest source of light. After flying for a time period essential for dealation, the alates drop to the ground and shed their wings, and the two sexes form tandem pairs with females in the lead (reviewed by Nutting, 1969).

Like most species of termites, the dealates of *C. formosanus* undergo distinct premating behaviors before constructing a nuptial chamber and mating. Tandem behavior was well studied by Buchli (1960) in *Reticulitermes lucifugus* Rossi. In this species, as in other *Reticulitermes* species, the females attain a calling posture by raising their abdomen. Males which are attracted to a calling female touch the latter with their antennae and mouthparts. This is followed by the formation of a tandem pair that searches for a suitable nest site. Whereas calling has not been observed in *Coptotermes* species, formation of tandem pairs is common in this genus. Buchli (1960) speculated that while a pheromone secreted by sternal glands may be involved in the initial attraction in *R. lucifugus*, a chemotropic factor produced continuously by the female accessory glands was perhaps responsible for subsequent attraction during tandeming. A trail pheromone produced by the sternal glands has been identified as *Z,Z,E-3*,6,8-dodecatrien-1-ol (DTE-OH) in *R. virginicus* (Banks) (Matsumura *et al.*, 1968) and *C. formosanus* (Tokoro *et al.*, 1989).

Laduguie *et al.* (1994) reported that in *R. santonensis* Feytaud, DTE-OH was 10 times more abundant in alates compared to workers and suggested that, at low concentrations, it acted as a trail pheromone and, at higher concentrations, as a sex pheromone. Wobst *et al.* (1999) made a similar claim for *R. lucifugus grassei* Clément. In *R. flavipes* (Kollar), *n*-tetradecylpropionate was described as the main component of the sex pheromone (Clément *et al.*, 1989).

The female imago in several species of termites has the epidermis of tergites 9 and 10 considerably thickened and differentiated into glands that have been referred to as tergal glands (Barth, 1955; Noirot, 1969). Barth (1955) observed in *Syntermes dirus* (Burmeister) that applying varnish to the last abdominal segments of the female prevented tandem behavior. Noirot (1969) suggested the production of a sex pheromone in termites by the tergal glands. In *Trinervitermes bettonianus* (Sjöstedt), only the female alates secrete sex pheromone from both sternal and tergal glands, with the latter providing the long-distance attraction (Leuthold, 1977). Recently, Bordereau *et al.* (2002) reported that pairing in *Cornitermes bequarerti* Emerson was mediated by a volatile sex pheromone secreted from the tergal glands of females. They identified the pheromone as DTE-OH, the same as the trail/sex pheromone identified from a number of termite species.

We examined the premating behavior in *C. formosanus*, including the role of male antennae and mouthparts, in maintaining tandem posture. We also explored the presence of a contact sex pheromone in the female dealates.

MATERIALS AND METHODS

Insect Collection

Light traps were placed at strategic places starting around the middle of April and until the first week of June of 2000–2002, in New Orleans, LA. Both alates and dealates in and around light traps and below street lights were collected with an aspirator and placed in plastic containers ($35 \times 25 \times 12.5$ cm, length \times width \times height) with moist paper towels. Behavior experiments were carried out the following day. In addition, a white sheet spread under a street light provided a platform to observe if lone females exhibited calling behavior and attracted males after dealation as well as tandem behavior.

Tandem Behavior

All behavior experiments were conducted under laboratory conditions (21 \pm 1°C, 60 \pm 5% RH, and 35 1ux). To determine the effect of wings on

tandem behavior, males and females were paired in 100×20 -mm petri dishes lined with filter paper, in four combinations: (1) males and females, both with intact wings; (2) dealated males with winged females; (3) dealated females with winged males; and (4) females with wings partially cut to expose the terminal four segments of the abdomen with dealated males. Tandem behavior was observed for six pairs in each treatment over a period of 5 min. A second experiment was conducted to determine whether the dealates (primary reproductives) resort to tandem behavior after mating. Virgin and mated (held in mating chambers for 5 days [Raina et al., 2003] males and females were paired in 100×20 -mm petri dishes in three combinations: (1) virgin male with mated female, (2) mated male with virgin female, and (3) mated male with mated female. Ten pairs were tested for each combination. Pairs were observed for 1 min every 30 min over a period of 8 h. Behaviors were categorized into three groups: no contact, short contact and grooming, and tandem pairing lasting for at least 1 min. After the experiment, females were dissected to determine the presence of sperm in the spermatheca. Data were subjected to analysis of variance (ANOVA) followed by Tukey's multiplecomparison test.

Ablation Experiment

From observations of tandem behavior it appears that antennae and/or other cephalic appendages in males may be involved in perception of the female. To assess the role of these appendages in tandem behavior, we selectively ablated these, with microscissors, from males that were chilled on ice to prevent bleeding. The experiment consisted of four treatments: (1) antennae removed at the base, (2) 10 terminal antennal segments (of an average of 20) removed, (3) maxillary palps removed, and (4) labial palps removed. In addition, there was an intact control group. One hour after ablation, males were paired with dealate virgin females in petri dishes ($100 \times 20 \text{ mm}$) and their behavior was observed for 5 min. Observations included time to first contact between male and female, time to initiation of tandem behavior, and percentage exhibiting tandem behavior. Ten pairs were observed for each treatment. The data were subjected to statistical analysis as described above.

Preparation and Analysis of Extracts

The three terminal segments of 50 female or 50 male abdomens were cut and placed on a filter paper under a dissecting microscope. All the internal organs and fat bodies were carefully removed until only the tergal

glands were left on the cuticle (present only in females under the 9th and 10th tergites). The tips were then extracted in 300 μ l hexane. The hexane extract was removed and the tips were rinsed again with 250 μ l hexane. The combined hexane extract was concentrated with nitrogen to 100 μ l. HPLC analyses were carried out with a Waters Alliance 2690 HPLC with a column heater and 996 Photodiode Array Detector. A Luna silica (2), 5- μ m, 2.0 × 150-mm (Phenomenex) column was used with a 20-min gradient from 1 to 50% ether in hexane, at a 0.3 ml/min flow rate. The procedure was repeated with extracts from both males and females.

Electrophysiology

Male dealates and female abdominal tip extracts were shipped overnight to the Chemicals Affecting Insect Behavior Laboratory in Beltsville, MD, for electrophysiology. Electrical responses of neurons associated with the long trichoid gustatory chemosensilla on antennae and maxillary palps of C. formosanus were obtained using a standard tip-recording technique (Hodgson et al., 1955). Whole-body preparations were used throughout, with adult termites affixed inverted on cork and held in place with tape. Antennae were pulled forward and immobilized with two-sided tape for easy access to chemosensilla on the terminal antennal segment. Maxillary palps were similarly immobilized for access to chemosensilla at the tip. A sharpened tungsten electrode inserted into the abdomen of the insect served as a reference electrode for all preparations. Silver wire inserted into a glass capillary, pulled and sized to fit over the tip of a single chemosensory hair, and filled with experimental chemicals in a solution of 0.01 M NaCl (90% distilled water and 10% ethyl acetate) served as a stimulating/recording electrode. Both electrodes were connected to a Grass P15D amplifier, and electrical responses were amplified and conditioned, viewed on an oscilloscope, monitored with a loudspeaker, and digitized for storage and analysis on a laboratory computer using Sapid (Smith et al., 1990) and AutoSpike (Syntech, Hilversum, The Netherlands) software. Mean numbers of spikes for various stimuli were compared using a two-tailed t test for unequal sample sizes (Ostle, 1963).

RESULTS

Swarming

The alates of *C. formosanus* swarm from April to June in Louisiana. Swarming started around 2000 h, and although most of the activity was over

within 1 h, some minor activity could be noticed almost until midnight. Alates flew to the nearest light source and, after hovering around it for some time, landed on the ground beneath. After landing the alates lost their wings and started forming tandem pairs. Alates that flew to light traps, kept at ground level, often did not lose their wings.

Tandem Behavior

Soon after swarming, large numbers of alates and dealates were seen on the ground below a light source. No calling activity was observed on the white sheet spread below the light. Males were not attracted to females even when the two were as close as 1–2 cm. Random encounters between a single male and a female resulted in their forming a tandem pair with female in the lead. Because of the usually large number of individuals on the ground, following a swarm, such accidental contact between the sexes occurred frequently. Tandem pairs were formed almost always only between dealated individuals. Occasionally up to four or five individuals were observed to form a chain in tandem, with additional males tagging to the one in front of them. This kind of tandem behavior did not last for more than few seconds.

In the laboratory experiment, when both males and females had intact wings, there was no tandem formation even if the two individuals came into contact with each other. The situation was similar when winged females were paired with wingless males. However, in rare cases a male stuck its head under the female's wings and established a brief tandem position (\bar{X} , 12.5 s; N=2). Winged males, on the other hand, readily formed tandem pairs with wingless females or with females whose wings had been partially cut to expose the terminal abdominal segments. Five of six pairs in each case continued in tandem during the 5-min observation period. With wingless males and females, all six experimental pairs continued tandem behavior during the 5-min observation. During the tandem behavior, when a female stopped, the male was observed to "lick" the terminal region of the female abdomen, using its palps and even mandibles. When the tandem behavior of three pairs was recorded for 5 h, two of the pairs were observed to stay in tandem continuously for >2 h.

When virgin males and previously mated females were paired, tandem behavior was observed in about 30% cases and 53% engaged in grooming (Table I). The highest incidence of tandem behavior (52%) was observed between mated males and virgin females. When previously mated males and previously mated females were paired, there was still tandem formation, although the incidence was significantly lower compared to the mated male and virgin female pairings (P < 0.001, Turkey's multiple-comparison test). Among this group, 77% were observed grooming. The spermatheca of all

 15.6 ± 3.9

Mated ♂ + mated ?

wated and virgin rema	Males showing types of contact (average $\% \pm SE$)			
Mated status	None	Grooming	Tandem	
Virgin σ + mated φ Mated σ + virgin φ	17.5 ± 2.4 6.9 ± 1.7	53.1 ± 4.0 41.3 ± 3.0	29.4 ± 2.9 51.8 ± 4.1	

Table I. Mated and Virgin Males Showing Various Behaviors When Paired with Previously Mated and Virgin Female Dealates of *Coptotermes formosanus* Over a Period of 8 h

Note. N = 10 pairs and each value is based on an average of 160 observations except for mated $\beta + \text{mated } \beta$, in which case N = 9 and the number of observations was 144. None—no contact; grooming—brief contact and grooming; tandem—tandem pairing for >1 min.

 76.9 ± 4.0

 7.5 ± 1.2

but one previously mated females contained sperm, thus confirming their mated status. The data for the lone unmated female were not included for statistical analysis.

Ablation of Antennae and Palps

Only males whose antennae were completely removed took significantly more time (P < 0.05, Dunnett's multiple-comparison test) to make first contact with the female and did not initiate tandem pairing (Table II). Ablation of 10 terminal antennal segments or the maxillary palps prolonged the time to first contact but the increase was not significantly different compared to control males. Likewise cutting of the labial palps had no effect on this parameter. Males with 10 terminal antennal segments or labial palps ablated took significantly longer to initiate tandem behavior (P < 0.01). Whereas 100% of control males established tandem pairs within the 5-min observation period, only 70–80% of males in the other three experimental groups (excluding the group with ablation of entire antennae) formed tandem pairs. Although 80% of the males with ablated labial palps formed tandem pairs,

Table II. Effect of Ablation of Antennae or Maxillary or Labial Palps in Males on Their Ability to Contact and Form Tandem Pairs with Females in Dealates of *Coptotermes formosanus*

	Time (means \pm SD) to			
Male status	First contact	Initiate tandem	Percentage forming tandem pairs	
Intact (control)	10.2 ± 3.5	26.6 ± 9.9	100	
Antennae ablated	$20.9 \pm 10.6^*$	_	0	
Half of antennae ablated	18.7 ± 11.4	$205.7 \pm 63.0**$	70	
Max. palps ablated	16.4 ± 7.2	73.6 ± 45.4	70	
Labial palps ablated	11.9 ± 6.0	$114.0 \pm 60.0^{**}$	80	

Note. *P < 0.05 and **P < 0.01, Dunnett's multiple-comparison test.

the behavior was erratic, and these males did not confine their contact to the terminal segments but appeared to explore different regions of the female's abdomen randomly.

Chemical Analysis of Extract

Hexane extracts of the tip of the male and female abdomen were analyzed using normal phase chromatography. All peaks in the chromatograms of the male extracts were also present in the female extracts. However, a unique peak was consistently observed in the chromatograms of the female extracts with a retention time of 11.0 min (Fig. 1).

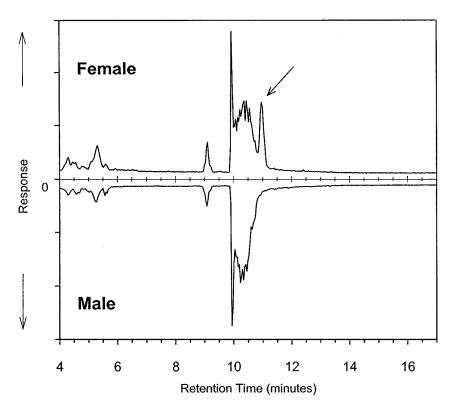


Fig. 1. Normal phase chromatograms of *Coptotermes formosanus* male and female abdominal tip extracts. Note that peaks in the chromatogram of the male extract are also present in the female extract except for a unique peak in the female extract with a retention time of 11.0 min.

Electrophysiology

Tip recordings from long gustatory trichoid sensilla on the tips of the antennae and maxillary palps revealed differential responses of neurons associated with each appendage to the female extract and solvent control. While responses to both the solvent control (0.01 M NaCl in 90% distilled water + 10% ethyl acetate) and the female extract were generally multineuronal, often the activity of a single spike was predominant (Fig. 2). Mean numbers of spikes elicited by the female extract were significantly greater than the number of spikes elicited by the control for both antennal and maxillary palp preparations (P < 0.05, two-tailed t test for unequal sample sizes (antennae—female extract, 18.4 ± 2.8 spikes/initial 500 ms ($\bar{X} \pm SE$; n = 19 sensilla on six males), and control, 6.8 ± 1.6 spikes/initial 500 ms (n = 21 sensilla on eight males); maxillary palps—female extract, 26.4 ± 3.5 spikes/initial 500 ms (n = 5 on two males), and control, 9.4 ± 2.6 spikes/initial 500 ms (n = 22 on six males)].

A. Control (0.01M NaCl)



B. Female tip extract



Fig. 2. Characteristic tip recording of electrical activity of neurons housed within a long trichoid sensillum near the tip of a maxillary palp of a male *Coptotermes formosanus*. (A) Response to solvent control (0.01 NaCl in 90% distilled water + 10% ethyl acetate). (B) Response to female abdominal tip extract in the above control solution. Each trace represents 500 ms following contact of electrode with tip of sensillum.

DISCUSSION

Formosan subterranean termites, like other subterranean termites, have a cryptic lifestyle, with only the period between swarming and entry into a nuptial chamber being open to observation. Premating behaviors are confined largely to this period. Because of the very large populations in mature colonies of *C. formosanus*, their swarms also contain thousands of alates. Swarming flights are primarily for dispersal, and neither courtship nor mating was observed during such flights. In nature, the alates after flying around the nearest light source for several minutes land on the ground and lose their wings. The pairs in tandem that survive heavy predation search for places suitable for colony formation. Fei and Henderson (1999) determined that the alates of *C. formosanus* preferred sites with high moisture availability to sites lacking moisture for colony formation. Once the pair finds a suitable area, they close it off and form a nuptial chamber. Subsequent behaviors leading to mating and formation of the incipient colony were observed under laboratory conditions and have been reported recently (Raina *et al.*, 2003).

Williams (1959) speculated that a pheromone which promotes dealation may be released by the females. Stuart (1969) suggested that a certain amount of flying was essential for dealation. Myles (1988) reported that dealation in *Pterotermes occidentis* (Walker) was highly dependent on the duration of flight. In this species, maximal rates of dealation were reached when alates had flown for ca. 17 min. However, in *Zootermopsis laticeps* (Banks) short flights, <3 min, were adequate to induce dealation (Myles, 1988). In *C. formosanus*, we did not determine the minimum time alates had to fly for effective dealation. Raina *et al.* (2003) reported that unflown alates of *C. formosanus* were able to form incipient colonies, as long as the pairs were artificially dealated.

Unlike females of *Reticulitermes* species, which call to attract males (Buchli, 1960; Stuart, 1969), no calling was observed in the case of *C. formosanus*, either in the field or in the laboratory. As a result, *C. formosanus* males could not locate a female even from the close distance of 1–2 cm. However, this may not be necessary because of the large number of individuals swarming at any given time and place, thus increasing the chance of accidental contact between sexes. Dealation, particularly in the case of females, was essential for establishing tandem pairs. The observation that a male could tandem, even briefly, with a female if it managed to get its head under the female's wings indicated that the dorsolateral area of the abdominal tip in the female was the attractive region. This was further confirmed when the wings of a female were partially cut to expose the three terminal abdominal segments. In this case the males formed tandem pairs which often lasted from several minutes to an hour.

It appears that the first contact by a male with a female involves the male antennae followed by the maxillary and labial palps. The contacts were made in the dorsolateral region of the terminal abdominal segments. Males with the entire antennae removed took significantly longer to locate a female but were never able to establish tandem pairs. Ablation of the terminal 10 segments of male antennae or removal of the maxillary or labial palps had no significant effect on the ability of the male to locate a female and form tandem pairs. However, ablation of half of the antennae or the labial palps resulted in significantly prolonging the time to tandem pairing. From these observations we suggest that the antennae are primarily responsible for locating a female using tactile and chemical cues, whereas the two types of palps, together with the antennae, may respond to a contact chemical. Our electrophysiological recordings from the long trichoid sensilla on both the antennae and the maxillary palps correlate well with the behavioral observations and support the idea that gustatory neurons on both appendages respond to chemicals in the female extract (Fig. 2).

Extracts of male and female abdominal tips showed almost identical HPLC profiles except for one peak that was evident only in the female extract. However, female abdominal tip extracts did not elicit male attraction in several bioassays that were tried. One possible explanation for the negative results may be that tactile cues from a live female are essential for attraction. Although there have been reports speculating the presence of a chemical sex pheromone in several species of termites (Buchli, 1960; Moore, 1969; Noirot, 1969), there is no previous report of such chemicals being present in Coptotermes species. We observed a pair of tergal glands in the 9th and 10th abdominal segments of female C. fomosanus. Identification of DTE-OH from the tergal glands of C. bequaerti led Bordereau et al. (2002) to speculate that this well-known trail pheromone acts as a sex pheromone in the adults of this species. In several Reticulitermes species, the greater abundance of DTE-OH in adults compared to workers (Laduguie et al., 1994; Wobst et al., 1999) tends to support the above hypothesis. As for the presence of DTE-OH in C. formosanus, tergal gland extracts, as well as solid phase microextraction preparations analyzed by GC-MS, did not reveal any trace of this compound. Even sternal glands in *C. formosanus*, the widely known source of DTE-OH in several termite species, are very much reduced in size in the adults compared to workers (unpublished results). Because the female-specific compound in the tergal gland extract did not show up in gas chromatographic analyses, it is possible that the compound is of a higher molecular weight. We speculate that such a nonvolatile compound produced in the tergal glands of *C. formosanus* may act as a sex pheromone that helps maintain contact during tandem behavior. Work is in progress to identify this chemical and determine its role in reproductive behavior and physiology.

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